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Characterization of a Human Monoclonal Antibody against Shiga Toxin 2 Expressed in Chinese Hamster Ovary Cells

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Shiga toxin-producing *Escherichia coli* infections can often lead to the development of hemolytic-uremic syndrome (HUS) in a small percentage of infected humans. Patients with HUS receive only supportive treatment as the benefit of antibiotic therapy remains uncertain. We have previously reported the generation and preclinical evaluation of neutralizing human monoclonal antibodies (HuMAbs) against the Shiga toxins (Stx). In this paper, we describe the expression in Chinese hamster ovary (CHO) cells of 5C12 HuMAb, which is directed against the A subunit of Stx2. The cDNAs of the light and heavy chain immunoglobulin (Ig) variable regions of 5C12 HuMAb were isolated and cloned into an expression vector containing human IgG1 constant regions. The vector was transfected into CHO cells, and transfectants secreting Stx2-specific antibody were screened by an Stx2-specific enzyme-linked immunosorbent assay. The CHO-produced recombinant 5C12 (r5C12) showed similar specificity and binding affinity to Stx2 as the parent hybridoma-produced 5C12. More significantly, the r5C12 displayed the same neutralizing activity as the parent 5C12 in vitro and in vivo. In the mouse toxicity model, both antibodies significantly and equally prolonged survival at a dose of 0.312 μg/mouse. The data showed that since r5C12, produced in CHO cells, was equally effective as the parent 5C12, it is our choice candidate as a potential prophylactic or therapeutic agent against hemolytic-uremic syndrome.

Approximately 110,000 cases of Shiga toxin-producing *Escherichia coli* infections are reported annually in the United States (26; reviewed in references 31, 36, and 55). Typical symptoms include abdominal pain and bloody diarrhea 2 to 5 days after exposure. While in the majority of cases the infection resolves after 10 to 14 days, in a small fraction of cases (5 to 10%), mostly in young children and the elderly, hemolytic-uremic syndrome (HUS) occurs, resulting in renal failure. Shiga toxin-producing *E. coli* strains produce two Shiga toxins, Stx1 and Stx2. Based on epidemiological studies, Stx2 production is a risk factor for the development of systemic complications including HUS (reviewed in references 5, 36, 39, 41, and 52). Both toxins possess an AB_5 structure, in which a single A subunit molecule is linked to five B subunit molecules. The A subunit contains the catalytic activity, an RNA *N*-glycosidase activity, while the B subunits are associated with binding to the host cell receptor, globotriaosyl ceramide [Gb_3; Galα(1-4)-Galβ(1-4)-Glcβ1-ceramide] (27, 31, 36, 55).

There are no preventive measures or specific therapeutics for HUS. Patients are given only supportive treatment as the use of antibiotics remains controversial and, in some studies, appears to increase the risk of HUS development (49, 59). Several therapeutic agents have been developed based on the concept that if the toxin(s) can be absorbed or neutralized in either the gastrointestinal tract or in the circulation, the development of HUS can be prevented. These agents include polymers of trisaccharide of Gb_3 (3, 11, 12, 20, 53, 58), carbosilane dendrimers with trisaccharides of Gb_3 located at their termini (SUPER TWIG[30]), bacteria with Stx-specific glycolipid receptors (34, 35), and Stx-specific antibodies (15, 18, 28, 29, 32, 37, 50). One limitation of most of these therapeutic agents, with the exception of antibodies, is that they are orally administered, which considerably reduces their efficacy. Large quantities of toxins produced by proliferating bacteria, which intimately attach to the cell membrane of epithelial cells in the gut, are difficult to intercept and to completely eliminate. Thus, continuous administration of large quantities of an antitoxin agent would also be required. In contrast, neutralization of the toxin in the blood circulation, close to the organ target, is much more efficient and requires only small quantities of an antitoxin agent, which in the case of antibody, has a relatively long half-life. We have previously reported the production and characterization of human monoclonal antibodies (HuMAbs) against Stx1 and Stx2 (28, 29) in transgenic mice which produce fully human antibodies in the absence of mouse antibodies (16, 23). These HuMAbs effectively neutralize the cytotoxic effects of the toxins in HeLa cells and are highly protective in both mice and gnotobiotic piglets (14, 28, 29, 46). The most efficacious of the Stx2-specific HuMAbs are those directed against the A subunit of Stx2, of which 5C12 is the most promising (28). Since these HuMAbs are administered intraperitoneally, they are anticipated to be more efficacious than the synthetic therapeutic agents.

While these human monoclonal cell lines have the potential to generate an almost unlimited supply of antibody, there are a number of limitations to the hybridoma technology, which can be overcome by production of these antibodies in alternate mammalian expression systems. There are also several disadvantages of using hybridoma cell lines including instability of high-producing cell lines, potential murine viral agents, and lack of flexibility in the generation of isotype variants or the production of Fabs or smaller antibody molecules without chemical cleavage. Production of recombinant antibodies and

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other glycoproteins in Chinese hamster ovary (CHO) cells is the most widely used mammalian expression system, particularly for production of human therapeutic agents for clinical trials (2, 8, 10, 42, 48, 54). The most commonly used CHO expression system is based on the use of CHO cells deficient in the production of endogenous dihydrofolate reductase (DHFR) coupled with a DHFR gene amplification system (1, 56, 57). These DHFR$^-$ CHO cells are transfected with either a single plasmid containing both antibody genes and a functional DHFR gene or two plasmids with the DHFR gene contained on a separate plasmid from the antibody gene cassettes (19, 33, 60). Transfected cells are selected in increasing concentrations of the drug methotrexate. Survival on high concentrations of methotrexate (1 to 10 μM) is associated with gene amplification of the DHFR gene during integration into the host chromosome or integration into active regions of the chromosome. During the DHFR gene amplification step, the antibody genes are also coamplified and integrated into the host chromosome. Antibody production levels as high as 20 to 110 pg/cell/day have been achieved when cells are grown in bioreactors or fermentors to high cell density (42, 54, 60).

In this paper, we report the production of a recombinant Stx2-specific HuMAb in CHO cells that has both in vitro and in vivo neutralization activity equivalent to the parent HuMAb produced in hybridoma cells. This recombinant HuMAb also showed similar specificity and binding affinity to Stx2 as the hybridoma-produced antibody. The modular nature of our mammalian expression vector allows for the flexibility to express any antibody as a human immunoglobulin G1 (IgG1), IgG2, IgG3, IgG4, Fab or F(ab')$_2$ molecule.

MATERIALS AND METHODS

Stx2 toxin. The production and purification of Stx2 toxin has been previously described (13). Lyophilized Stx2 toxin was provided by Anne Kane (New England Medical Center, Boston, MA).

Human anti-Stx2-producing hybridoma cells. Murine hybridomas secreting Stx2-specific HuMAbs have been previously described (28, 46). The 5C12 HuMAb, an anti-Stx2 A subunit-specific antibody of the IgG1(κ) isotype, was shown to be one of the most effective HuMAbs in neutralizing Stx2 both in vitro and in vivo. The 5C12 HuMAb-secreting hybridoma was grown in serum-free medium (HyQ ADCF MAb; HyClone, Logan, UT) in CL1000 CELLine Bioreactors (Integra Biosciences, Inc., East Dundee, IL). The medium was harvested every 7 days, the cells were removed by centrifugation, and the supernatant was passed through a 0.22-μm filter. This supernatant, which contained the antibody fraction, was used without further purification in this study. The antibody was quantitated by a human IgG1 enzyme-linked immunosorbent assay (ELISA), and the yield was approximately 200 μg/ml (2 to 3 mg/harvest). For reference, this antibody will be referred to as the parent 5C12 HuMAb.

Sequence determination of the 5C12 heavy and light chain variable regions. Total RNA was extracted from the 5C12-producing hybridoma cell line (2×10^6 cells) using the RNeasy Kit (QIAGEN, Inc., Valencia, CA) following the manufacturer's protocol. To amplify the 5C12 heavy chain variable region (V_H), three degenerate sense primers (HuIgV$_H$5'-A, -B, and -C; Novagen Inc., Madison, WI) and one antisense primer were used. The sense primers are located within the leader sequences and should amplify all the human heavy immunoglobulin subgroups. The single antisense primer (AB90) binds within the constant (C) gamma (1 or 3) C$_H$1 region. To amplify the variable regions of the different kappa light chains (V_κ), a single degenerate sense primer located within the leader sequence (HuIgκV$_\kappa$5'-A; Novagen) and an antisense primer (HuIgκV$_\kappa$3'-1; Novagen) located within the C kappa region, were used. The sequences of these primers are as follows: HuIgV$_H$5'-A, 5'-GGGAATTCATG GACTGGACCTGGAGGRTCYTCTKC-3'; HuIgV$_H$5'-B, 5'-GGGAATTCAT GGAGYTTGGGCTGASCTGGSTTTYT-3'; HuIgV$_H$5'-C, 5'-GGGAATTCA TGRAMMWACTKTGKWSCWYSCTYCTG-3'; AB90, 5'-TGCCAGGGGGA AGACCGATGG-3'; HuIgκV$_\kappa$5'-A, 5'-GGGAATTCATGGACATGRRRDYC CHVGYKCASCTT-3'; and HuIgκV$_\kappa$3'-1, 5'-CCCAAGCTTCATCAGATGGC GGGAAGAT-3'.

Using standard PCR technology, reactions were set up using High-Fidelity PCR Master Mix (Roche Diagnostics Corp., Indianapolis, IN). The PCR program used for all reactions was 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. An aliquot of each reaction was run on a 1.5% Tris-acetate-EDTA agarose gel to determine if a band in the 400- to 550-bp range was present. PCRs showing the strongest intensity bands within this expected size range were cloned into the pCR4-TOPO vector (Invitrogen Corp., Carlsbad, CA) and sequenced. A minimum of four clones was sequenced from each PCR. To eliminate possible PCR artifacts, two independent RNA isolations and PCRs were performed for each variable region.

Mammalian expression vectors. The two mammalian expression vectors, VHExpress and VKExpress (38), were obtained from Invitrogen. These vectors were used as the backbone for our antibody expression cassettes (VHExpress) and for the DHFR gene cassette (VKExpress). The backbones of these two vectors are identical except for the eukaryotic-selectable antibiotic marker and are modified to eliminate specific restriction sites. The vectors contain the following components: colE1 and f1 bacterial origins of replication, simian virus 40 (SV40) origin of replication for transient expression in eukaryotic cells, β-lactamase gene for bacterial selection, and a neomycin phosphotransferase gene (VHExpress) or xanthine-guanine phosphoribosyltransferase gene (VKExpress) for eukaryotic selection (Fig. 1).

The IgG1 expression vector was constructed to be modular in nature such that different V_H and variable light chain (V_L) regions can be cloned into unique restriction sites. The 5C12 HuMAb was expressed as an IgG1 molecule, using an expression system in which the heavy and light chains were separately expressed from the cytomegalovirus (CMV) promoter. The CMV promoter was amplified from pCMV-Script plasmid (Invitrogen) using a standard protocol for site-specific mutagenesis by overlap extension PCR (44) in order to mutate an internal NcoI site within the CMV promoter. The IgG1 heavy and light chain leader sequences were derived from another Stx2-specific HuMAb (5H8) (28), using 5' rapid amplification of cDNA ends (RACE) methodology (Invitrogen). The heavy and light chain constant regions were also derived from the 5H8 HuMAb using 3' RACE technology (Invitrogen). Unique restriction sites were engineered within the leader sequences and at the 5' ends of the constant regions to permit the cloning of different V_H and V_κ sequences (Fig. 1). Using standard PCR methodology, these same restriction sites were added to the heavy and light chain variable regions of our different HuMAbs. The expression vector which produces the 5C12 HuMAb in CHO cells was designated p5C12IgG1.

Construction of pdhfrExpress. The DHFR expression cassette contained the mouse DHFR gene expressed from the mouse β-globin major promoter and the SV40 polyadenylation (pA) signal sequence. The β-globin major promoter (GenBank accession no. J00413) was amplified from genomic DNA isolated from mouse liver tissue. The β-globin Kozak sequence was also modified to create a consensus Kozak sequence (21). The mouse DHFR gene was amplified from the pSV2-dhfr vector (ATCC 37146) (51) and was modified such that the start codon (ATG) was contained within an NcoI site. The SV40 pA signal sequence was amplified from pCMV-Script plasmid DNA (Invitrogen). The assembled DHFR gene cassette contains a unique EcoRI site upstream of the promoter cassette and a KpnI site downstream of the SV40 pA signal sequence. These two restriction sites were used to clone the DHFR expression cassette into the VKExpress vector (38) digested with EcoRI/KpnI to generate pdhfrExpress (Fig. 1B).

Transfection into CHO cells. The CHO double DHFR$^-$ mutant cell line, DG44, was kindly provided by L. Chasin (Columbia University, New York, NY) (56, 57). The nontransfected DG44 cells were maintained in minimum essential medium α (αMEM) with ribonucleosides and deoxyribonucleosides and dialyzed fetal calf serum (dFCS; 10% [vol/vol]; Invitrogen). The two plasmids, p5C12IgG1 (3 μg) and pdhfrExpress (150 ng), were cotransfected into DG44 cells grown as a monolayer (80 to 90% confluent in 25-cm^2 flasks under 5% CO_2) using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. The cells were allowed to recover for 24 h before the medium was switched to αMEM without ribonucleosides and deoxyribonucleosides but containing dFCS (10%), which was used for the subsequent selection. The cells were split (1:2) into medium containing G418 (200 μg/ml; Invitrogen) and methotrexate (5 nM; Sigma-Aldrich, St. Louis, MO). The surviving cells were subsequently grown in medium containing G418 (500 μg/ml) and increasing concentrations of methotrexate (up to 500 nM) over a 4-month period. At this point, the cells were adapted to serum-free medium [CHO III(A); Invitrogen], supplemented with 0.7% dFCS, G418 (500 μg/ml), methotrexate (500 nM), and L-glutamine (2 mM; Invitrogen). During the selection process, the production of antibodies was

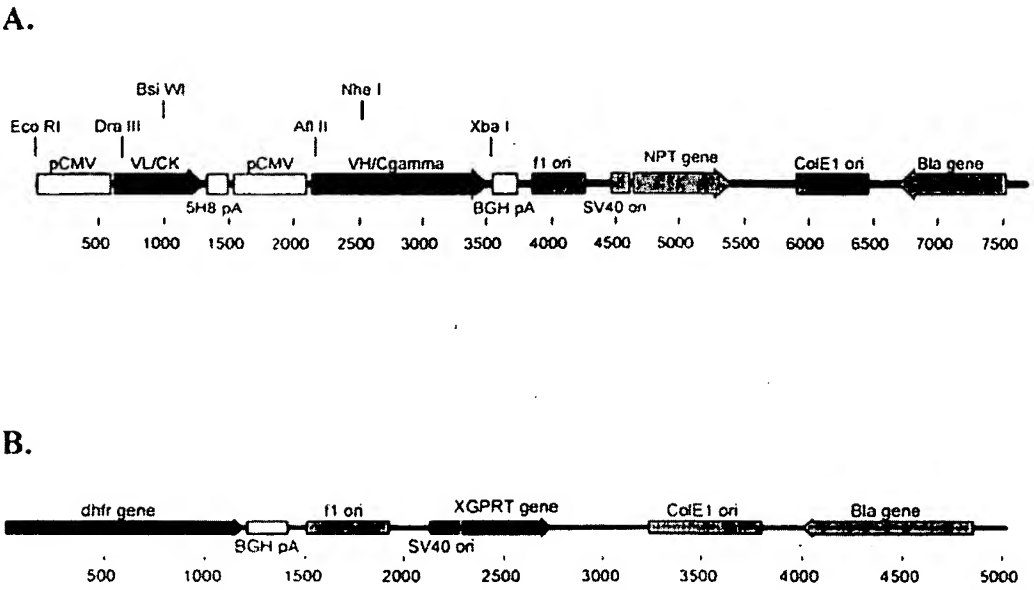

FIG. 1. Schematic of the p5C12IgG1 and pDHFRExpress vectors for the expression of recombinant HuMAbs in CHO cell lines using a DHFR-CHO gene amplification system. (A) The pIgG1 expression vector for the expression of Stx2-specific HuMAbs. VL/CK, variable and constant region of the κ light chain; VH/Cgamma, variable and IgG1 constant region of the heavy chain; pCMV, CMV promoter; 5H8 pA, polyadenylation signal from the 5H8 HuMAb light chain; BGH pA, polyadenylation signal from bovine growth hormone gene; f1 ori, origin of replication from f1; SV40 ori, SV40 origin of replication; NPT gene, neomycin phosphotransferase gene; ColE1 ori, origin of replication from ColE1; Bla gene, β-lactamase gene. (B) The pDHFRExpress vector, which has a functional mouse DHFR gene in place of the antibody expression cassettes and the xanthine-guanine phosphoribosyltransferase (XGPRT) instead of the NPT gene, but otherwise is identical to the p5C12IgG1 vector. The DHFR gene is expressed from the β-globin major promoter.

measured by an Stx2 or IgG1 ELISA. The recombinant 5C12 (r5C12)-producing CHO cell line was designated CHO-5C12 IgG1#16.

Scaled-up production of r5C12 HuMAb. In order to produce larger quantities of r5C12 HuMAb, CHO-5C12 IgG1#16 was scaled up to a CELLine AD 1000 Bioreactor (Integra Biosciences), which is specifically designed for adherent cell cultures. The culture supernatant was harvested every 7 days (four harvests per bioreactor), clarified by centrifugation, and filtered through a 0.22-μm filter. The yield of antibody ranged from 2 to 6 mg/harvest. For reference, this antibody will be referred to as r5C12.

Comparison of HuMAb production in the CHO and hybridoma cell lines. The CHO-5C12 IgG1#16 and the 5C12 HuMAb-secreting hybridoma cell lines were seeded at a density of 3×10^5 cells/ml in 75-cm^2 flasks in duplicate. The CHO cells were grown as suspension cultures in HyQ PF CHO Liquid Soy (HyClone) and the hybridoma cells were grown in HyQ ADCF MAb, which are both protein- and serum-free media. An aliquot (0.5 ml) was removed every 24 h from each flask, and the cell number and IgG1 concentration were determined over a period of 5 days.

Stx2-specific ELISA. The r5C12 and parent 5C12 HuMAbs were assayed for their ability to bind to Stx2 toxin. Ninety-six-well plates (9018; Costar, Corning, NY) were coated overnight with Stx2 toxin (1.5 μg/ml in phosphate-buffered saline [PBS]) at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and then blocked with 1% bovine serum albumin (BSA; 100 μl; Sigma-Aldrich) for 1 h at 37°C. After a wash step, a dilution series of the r5C12 and parent 5C12 HuMAbs was made by diluting the antibodies to 1 μg/ml in PBS-BSA and serially diluting twofold in PBS-BSA in duplicate rows of the plate (100 μl/well). The plates were incubated at 37°C for 1 h. Following PBS-T washes, horseradish peroxidase-conjugated goat anti-human IgG (tested for minimal cross-reactivity against mouse, bovine, or horse serum proteins) (Pierce Biotechnology, Inc., Rockford, IL) was added (1:5,000 dilution; 100 μl/well), and the plates were incubated at 37°C for 1 h. Following PBS-T washes, plates were developed with a substrate solution (0.2% o-phenylenediamine, 0.05% hydrogen peroxide in citric acid-phosphate buffer, pH 5). The colorimetric reaction was stopped by the addition of 3 M sulfuric acid (50 μl), and the absorbance was read at 490 nm. Blank wells contained PBS instead of sample and were similarly processed as the sample wells.

IgG quantitation ELISA. The quantitation of the r5C12 and parent 5C12 HuMAbs was by IgG ELISA. The 96-well plates were coated with goat anti-human IgG antibody (1 μl/100 μl/well; IgG-Fc) (Bethyl Laboratories, Inc., Montgomery, TX) and the assay was carried out as described for the Stx2-specific ELISA, with the exception that the secondary antibody was a horseradish peroxidase-conjugated goat anti-human IgG-Fc (1:5,000 dilution; Bethyl Laboratories). A human IgG1(κ) standard (Sigma-Aldrich) and the IgG standard included in the Bethyl ELISA kit were used interchangeably at a starting concentration of 1 μg/ml and were serially diluted twofold in duplicate wells. Blank wells contained PBS instead of sample and were similarly processed as the sample wells.

Vero cell cytotoxicity assay. The r5C12 HuMAb was evaluated for its ability to neutralize the cytotoxic effects of Stx2 against Vero cells compared to that of the parent 5C12 HuMAb. Briefly, Vero cells (ATCC CCL-81) were seeded at 1.1×10^4/well in 96-well plates in αMEM (Invitrogen) containing 5% fetal bovine serum (Invitrogen) and 2 mM glutamine, and incubated overnight at 37°C in 5% CO_2. The titer of the Stx2 preparation was first ascertained to determine the concentration which resulted in approximately 75% killing of the Vero cells. Briefly, Stx2 was serially diluted twofold in medium, starting at a concentration of 200 ng/ml, and each dilution was assayed in triplicate. The toxin (100 μl) was then added to the Vero cells and incubated for 48 h at 37°C in 5% CO_2. The wells were then extensively washed with PBS to remove dead and detached cells. The remaining cells were fixed in 2% formalin (100 μl/well) for 10 min. Following removal of the formalin solution, crystal violet solution (100 μl/well; 0.13% crystal violet, 5% ethanol, 2% formalin in PBS, pH 7.4) was added and incubated for 5 min. The plates were washed with water, and ethanol was added (100%; 100 μl/well) and incubated for 5 min. The wells were mixed thoroughly before absorbance was read at 590 nm. The Stx2 concentration resulting in 75% killing was determined based on this toxin kill curve.

The r5C12 and parent 5C12 HuMAbs were assayed by twofold serially diluting these antibodies in medium across a 96-well plate row from a starting concentration of 500 ng/ml (100 μl/well in medium) down to 15 ng/ml. Stx2 (100 μl; 50 ng/ml; concentration determined to give 75% kill) was added to each well containing antibody. The antibody-toxin mixtures were incubated at 37°C for 1 h and then added to the Vero cells and incubated at 37°C in 5% CO_2 for 48 h. Each antibody dilution was tested in triplicate. Control wells contained Vero cells only or Vero cells plus Stx2 (100 μl; 50 ng/ml), and blank wells contained medium only (in triplicate). The plates were processed as described above for the Stx2 kill curve assay. Two independent assays were performed, with each antibody concentration tested in triplicate. An Stx2 kill curve (200 ng/ml to 0.78 ng/ml) was included on each 96-well plate.

Murine Stx2 toxicity model. The murine Stx2 toxicity model (18, 28, 29, 32, 50) was used to compare the neutralization ability of the r5C12 HuMAb to that of

the parent 5C12 HuMAb in vivo. A dose-response study was performed with groups of 10 or 20 3- to 4-week-old female Swiss-Webster mice (15 to 25 g; Taconic, Germantown, NY) to evaluate efficacy. The r5C12 or parent 5C12 HuMAb at doses of 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, or 5 μg/mouse in 200 μl of PBS was administered intraperitoneally (i.p.). Stx2 (72 ng in 200 μl of PBS) was administered i.p. 4 h later. A control group of 20 mice received human IgG1(κ) (5 μg/mouse; Sigma-Aldrich). Mice were observed three times per day for survival. The survival data was analyzed by both parametric (log rank test) and nonparametric (Wilcoxon test) methods using the Prism 4 software program (version 4.0; GraphPad Software, Inc., San Diego, CA). Comparable *P* values were obtained with both methods. *P* values of <0.05 were considered significant. All mouse experimental procedures were approved by the Tufts University School of Veterinary Medicine Institutional Animal Care and Use Committee.

RESULTS

Sequence determination of the immunoglobulin variable region genes of the parent 5C12 HuMAb. Total RNA was isolated from hybridoma cells secreting 5C12 HuMAb, and the V_H and V_κ cDNAs were obtained by reverse transcriptase PCR. The amplified V_H and V_κ genes were cloned into the pCR4-TOPO vector and sequenced. The variable region genes of 5C12 were aligned to other published human immunoglobulin genes using DNAPLOT for V BASE sequences (www.mrc_cpe.cam.ac.uk/DNAPLOT) or to GenBank sequences using the Immunoglobulin BLAST search tool (www.ncbi.nlm.nih.gov/BLAST). Based on sequence similarity, the variable region of the 5C12 light chain belonged to the human κ subgroup III and differed from the L6 locus (EMBL X01668) by only a single base. The variable region of the 5C12 heavy chain belonged to the human IgG1 class III subgroup and differed by seven bases from the VH3-30.3 locus sequence (Z12346). The L6 locus is contained on KCo5 and VH3-30.3 is on HCo12, both of which are among the variable genes included in the HuMAb mouse transgenes (16, 23).

Sequence determination of the 5H8 leader and constant regions. Both 5′ and 3′ RACE technologies was used to determine the leader sequences and constant regions of the 5H8 HuMAb heavy and light chains, respectively, which were subsequently incorporated into the design of the CHO expression vector.

Construction of the CHO expression vector. A two-plasmid expression system was used to express human recombinant antibodies against the Shiga toxins. The first plasmid (p5C12IgG1) contained both the light and heavy chain expression cassettes, while the second plasmid, pdhfrExpress, contained the DHFR gene cassette. The light and heavy chains were expressed separately from the CMV promoter in order to produce equal quantities of light and heavy chains. The light and heavy chain leader sequences and constant regions from 5H8, another of our HuMAbs, were incorporated into the vector backbone of our expression vector. Using PCR technology, unique restriction sites were engineered within the leader sequences and constant regions (Fig. 1) to allow for different variable regions to be cloned in after they had been modified to contain the same restriction sites. The heavy chain constant region of p5C12IgG1 can also be easily replaced with constant region cassettes of IgG2, IgG3, and IgG4, as well as with a truncated fragment containing only C_H1, for the production of Fab fragments using the unique restriction sites engineered into the design of the vector. More recently, we have switched to a single vector expression system whereby the DHFR gene was cloned onto the p5C12IgG1 vector at the unique EcoRI site (Fig. 1). This single vector expression system appeared to give higher transfection frequencies, and selection in methotrexate (from 0 to 500 nM) was reduced to about 6 weeks (data not shown).

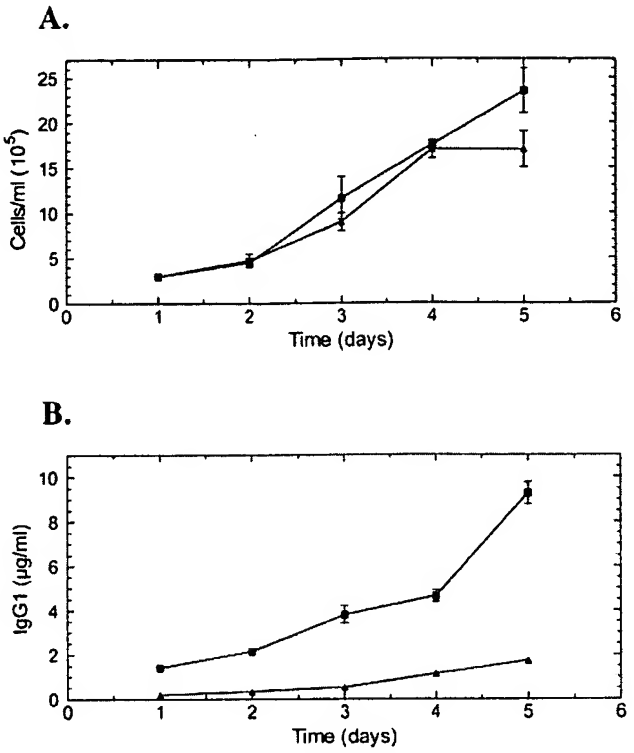

FIG. 2. 5C12 production in the CHO (■) and hybridoma (▲) cell lines. Both cell lines were grown as suspension cultures in 75-cm^2 flasks in duplicate and were monitored over a 5-day period. (A) Each flask was initially seeded at a density of 3×10^5 cells/ml. Viable cell number was determined by trypan blue exclusion and cells were counted using a hemacytometer. (B) IgG1 production was measured by the IgG quantitation ELISA. Each error bar represents the standard error of the mean.

The p5C12IgG1 and the pdhfrExpress vectors were cotransfected into DG44 cells. Transfectants were initially selected in medium lacking ribonucleosides and deoxyribonucleosides but containing G418 (200 μg/ml). Methotrexate was initially added at a concentration of 5 nM and was subsequently increased to 500 nM over a period of 4 months. Secretion of full-length HuMAb was quantitated by a human IgG ELISA, using a horseradish peroxidase-conjugated secondary antibody against the Fc region. Specificity of the secreted antibodies was determined using a Stx2-specific ELISA. The highest-producing stable cell line was 5C12 IgG1#16, which produced 10 μg over a 2- to 3-day period in 25-cm^2 flasks.

For the production of larger quantities of r5C12 HuMAb for testing and characterization purposes, the 5C12 IgG1#16 cells were grown in CL1000 AD Bioreactors, and supernatant was harvested every 7 days over a period of a month. The 5C12 IgG1#16 cell line was recently adapted to serum- and protein-free suspension medium (HyQ PF CHO Liquid Soy). To compare production of 5C12 IgG1#16 and the hybridoma-produced 5C12 cells under similar growth conditions, both cell lines were grown as suspension cultures in 75-cm^2 flasks. The cells were seeded at a density of 3×10^5 cells/ml, and viable cell number and amount of IgG1 were determined daily over a period of 5 days (Fig. 2). The number of viable cells and the

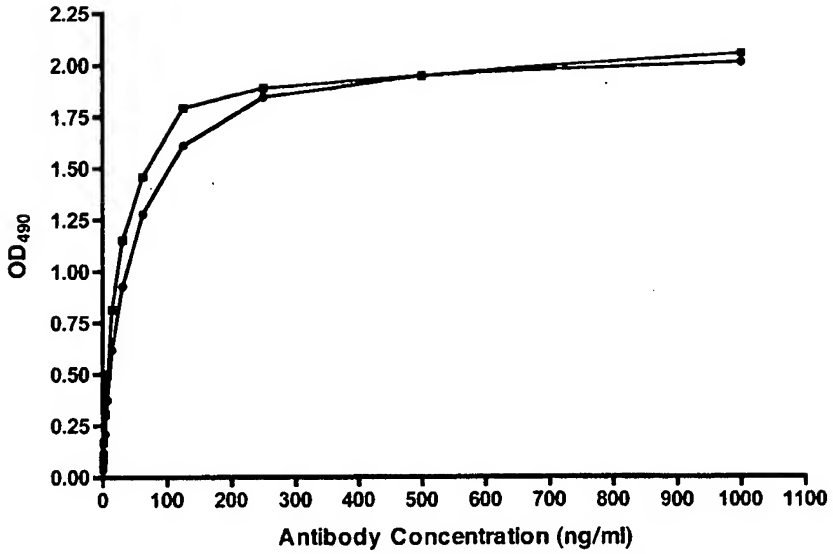

FIG. 3. Comparison of the binding affinity and specificity of r5C12 (■) to the parent 5C12 (●) using an Stx2-specific ELISA. Both antibodies were diluted twofold from 1 μg/ml to 0.4 ng/ml. Plates were coated with Stx2 and blocked, and 5C12 HuMAb (r5C12 or the parent 5C12) was added. Anti-human IgG-Fc coupled to horseradish peroxidase was added, the plates were developed with o-phenylenediamine solution, the colorimetric reaction was stopped by the addition of sulfuric acid, and the optical density was measured at 490 nm (OD_{490}). Each data point represents the average of four replicates (two independent assays). The standard deviation of duplicate data points for each assay was <0.075.

growth rates of the two cultures were similar during this time period (Fig. 2A). For each day, the amount of r5C12 in the CHO cells was about four to seven times higher than that of the 5C12-secreting hybridoma cells at similar cell numbers (Fig. 2B).

Binding affinity of r5C12 HuMAb. The binding affinity of the r5C12 to Stx2 was compared to that of the parent 5C12 HuMAb using the Stx2-specific ELISA. The r5C12 and parent 5C12 HuMAbs were serially diluted twofold from 1 μg/ml to 0.4 ng/ml, with each dilution tested in duplicate, and two independent ELISAs were performed. The optical density of each well was measured as absorbance, and the quadruple readings for each dilution were averaged (Fig. 3). The binding affinity curves of the r5C12 and parent 5C12 HuMAbs were not significantly different, indicating that the binding affinity of r5C12 was similar to that of its parent 5C12 HuMAb.

Neutralization of Stx2 in the Vero cytotoxicity assay. The relative activity of r5C12 HuMAb to neutralize the toxic effects of Stx2 on Vero cells was studied. The percent of Stx2 (5 ng; determined to kill 75% of the Vero cells) neutralized by various amounts (50 to 1.6 ng; twofold dilutions) of r5C12 or the parent 5C12 HuMAb was measured. The neutralization data for the two HuMAbs are summarized in Fig. 4 and represent the mean percent neutralization of triplicate wells from two independent assays. The results showed that both antibodies had similar Stx2 neutralization curves. Seventy-five percent of the toxin (e.g., 3.75 ng) was neutralized by 12.5 ng of r5C12 or the parent 5C12 HuMAb. The percentage of neutralization of toxin dropped to approximately 50% and 15% at antibody levels of 6.3 ng and 1.6 ng, respectively. Similar results were observed when HeLa cells (ATCC CCL-2) were used instead of Vero cells.

In vivo mouse Stx2 toxicity model. Previously published work indicated that not all Stx2-specific HuMAbs, which neutralized well in the HeLa cell cytotoxicity assay, significantly prolonged survival in the in vivo mouse toxicity model (28). Therefore, the activity of the r5C12 HuMAb was evaluated in the mouse toxicity model to determine if prolonged survival was observed in a dose-dependent manner similar to the parent 5C12 HuMAb. The mice were given HuMAb (0.078 to 5 μg/mouse) i.p. 4 h prior to Stx2 (i.p.; 75 ng/mouse, which is equivalent to ~75 times the 50% lethal dose), and control mice received human IgG1(κ) (5 μg/mouse). Using the log rank test, both 5C12 HuMAbs significantly prolonged survival (experiments were terminated on day 7) compared to the human IgG1(κ) control group, which had a mean survival of 3.2 days

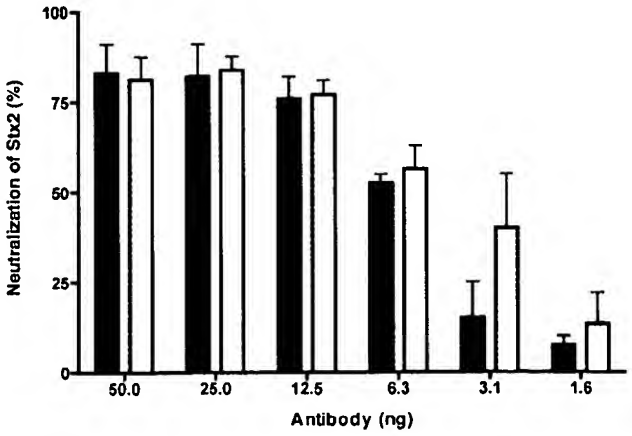

FIG. 4. Neutralization of Stx2 in a Vero cytotoxicity assay. The percent neutralization of 5 ng of Stx2 by various amounts (1.6 to 50 ng) of r5C12 (filled bars) or the parent 5C12 (open bars) was measured using a Vero cytotoxicity assay. Neutralization curves from two independent assays (triplicate wells) were determined for each antibody and used as a basis for the mean percent neutralization values shown here. Each error bar represents the standard error of the mean.

TABLE 1. Neutralization activities of the r5C12 and the parent 5C12 HuMAbs against Stx2 in the mouse toxicity model

Antibody and dose (μg/mouse)	Survival rate (no. of surviving mice/total no. of mice [%])[a]	No. of days (mean ± SD)[b]	P	
			Control group vs treated group[c]	r5C12 group vs 5C12 group[d]
Control	1/20 (5)	3.20 ± 1.005[e]		
r5C12				
0.078	0/10 (0)	3.50 ± 0.527	0.1247	0.1704
0.156	0/10 (0)	3.70 ± 1.059	0.3191	0.5489
0.312	11/20 (55)	5.60 ± 1.789	<0.0001	0.0132
0.625	13/20 (65)	5.80 ± 1.735	<0.0001	0.9416
1.25	18/20 (90)	7.00 ± 0.0[f]	<0.0001	0.3173
2.5	10/10 (100)	7.00 ± 0.0	<0.0001	0.3173
5.0	10/10 (100)	7.00 ± 0.0	<0.0001	0.3173
Parent 5C12				
0.078	0/10 (0)	3.20 ± 0.422	0.7078	
0.156	2/10 (20)	3.80 ± 1.687	0.2060	
0.312	4/20 (20)	4.30 ± 1.559	0.0065	
0.625	13/20 (65)	5.60 ± 1.957	<0.0001	
1.25	20/20 (100)	7.00 ± 0.0	<0.0001	
2.5	9/10 (90)	6.60 ± 1.265	<0.0001	
5.0	9/10 (90)	6.70 ± 0.949	<0.0001	

[a] Number (percentage) of mice that survived through day 7 and were euthanized.
[b] Experiments were terminated on day 7. Mice euthanized on day 7 were given a survival score of 7 days.
[c] *P* values were calculated for the comparison of the mean survival of the control group and each HuMAb-treated group by parametric (log rank test) and nonparametric (Wilcoxon) analyses. Comparable *P* values were obtained with both analyses. *P* values shown in the table were calculated by the log rank test.
[d] *P* values comparing mean survival of r5C12 versus the parent 5C12 groups at the same antibody doses.
[e] Nineteen of 20 mice in this group died between days 2 and 4; 1 mouse survived.
[f] One mouse died on day 7.

(Table 1). Significant prolonged survival was observed for both antibodies down to a dose of 0.312 μg/mouse. Prolonged survival was not observed at the two lower doses of 0.078 and 0.156 μg/mouse. The survival curves of the antibody-treated groups corresponding to the same dose of either r5C12 or the parent 5C12 HuMAb were also analyzed, and except for the 0.312-μg/mouse group ($P = 0.0132$), the difference in the mean days of survival was not significant ($P > 0.05$) between the matched dosage groups. The data clearly demonstrated that the r5C12 HuMAb showed protection equivalent to that of the parent 5C12 HuMAb.

Nineteen of the 20 control mice, which received no antibody, died between days 2 and 4. Survival of >50% of the mice was observed in the antibody-treated groups at antibody doses as low as 0.312 μg/mouse (r5C12) and 0.625 μg/mouse (parent 5C12). Both of these groups had a mean survival of >5 days. At antibody doses of ≥1.25 μg/mouse, survival rates of 90 to 100% were observed for both antibody groups, indicating the effectiveness of both antibodies to protect mice from the cytotoxic effects of the toxin.

DISCUSSION

The primary goal of this study was the production in CHO cells of 5C12 IgG1, the Stx2 HuMAb selected for preclinical evaluation. The long-range goal is to utilize this antibody-producing CHO cell line to obtain large amounts of antibody for clinical use. From a production viewpoint, the 5C12-producing CHO cell line had a four- to sevenfold higher production compared to the 5C12-producing hybridoma under similar growth conditions. Using recombinant DNA technology, the DNA sequences of the immunoglobulin variable regions of the parent 5C12 HuMAb were determined and expressed as a human IgG1 antibody using a CHO expression vector. Using a CHO-DHFR gene amplification system, one CHO cell line was selected that produced 5C12 HuMAb and was subsequently scaled up to produce sufficient quantities of antibody for testing. The r5C12 HuMAb showed the same specificity and affinity for purified Stx2 as the parent 5C12 HuMAb when tested in an Stx2-specific ELISA. More importantly, the r5C12 HuMAb neutralized Stx2 as effectively as the parent 5C12 HuMAb both in vitro and in vivo. At antibody doses of 0.312 μg/mouse or lower, both antibodies protected mice from the toxic effects of Stx2 and showed similar dose-response curves. Given the equivalent neutralizing efficacy, r5C12 can now be used for large-scale production for clinical purposes, which was the main objective of this study. The next step will be to generate a recombinant 5C12 Fab and to determine if it has the same neutralizing activity as the r5C12.

There are a number of advantages to producing HuMAbs in CHO cells compared to hybridoma cells. The first is the ability to adapt CHO cells to serum-free, protein-free suspension cultures and to grow these to relatively high cell densities in bioreactors or fermentors. A second advantage is the ability to select for high-antibody-producing transfected CHO cells through screening and selection using methotrexate. Using high-throughput flow cytometry, thousands of transfectants can be quickly screened early in the selection process to select high-producing clones (7, 61). Production levels can be further increased by increasing methotrexate concentrations up to 1 to 5 μM. Another advantage, particularly if the antibody is to be used as a human immunotherapeutic agent, is the absence of murine adventitious viral agents. Also, since the antibody produced in CHO cells is recombinant in nature, it can be subsequently genetically engineered. These HuMAbs can be produced as IgG1, IgG2, IgG3, or IgG4 isotypes, depending on the application. They can also be produced in CHO cells as Fab or $(Fab')_2$ molecules, if effector functions are not required. Furthermore, the binding affinities of recombinant antibodies can be modified using affinity maturation technology (reviewed in reference 25). There may also be some disadvantages in using a CHO expression system to produce antibodies. The glycosylation pattern of antibodies produced in CHO cells is such that these antibodies are under-glycosylated and under-sialylated and lack the addition of bisecting *N*-acetylglucosamine compared to native human antibodies (40). However, human antibodies secreted by hybridoma cells also have a different glycosylation pattern compared to human antibodies produced in human cells. Most notable is the addition of Gal(α1-3)galactose, which humans have antibodies against. The consequences of these glycosylation differences on the pharmacokinetics of an antibody and generation of an immune response to the carbohydrate moiety are unclear, but there are reports which suggest the absence of an immune response to proteins produced in CHO cells (17) or in hybridomas (24).

While production of antibodies in either hybridoma or CHO cells is well documented, one issue continues to be a problem

for both systems: the ability of a cell line to maintain a constant level of protein production over a prolonged period of time (reviewed in reference 6). The general conclusion is that both types of cells are reasonably unstable with respect to production levels during long-term culture. In hybridomas, decreased production of antibodies is often observed and is associated with the presence of a nonproducing population, which usually has a slightly faster growth rate. Production of heterologous proteins in DHFR$^-$ CHO cells also decreases over time, particularly in the absence of methotrexate selection, due presumably to the loss in copy number of the recombinant gene. The site of integration within the genome is also a critical factor for stability, and several papers report that integration of recombinant genes near the telomeric regions is more likely to be stable. Whether or not a specific antibody will be more stable in CHO versus hybridoma cells must be experimentally determined. A recent paper describes the stable expression of a human monoclonal antibody in CHO cells compared to the same antibody secreted by a human-mouse heterohybridoma cell line, where poor stability was observed (4, 9).

The production of recombinant proteins for therapeutic applications, including antibodies, in CHO cells and *E. coli* will continue to be the two primary expression systems. Several challenges lie ahead before either of these systems is optimum for expression. The major issues associated with using *E. coli* as an expression system are lack of glycosylation of proteins and efficient refolding of whole antibody molecules to produce functional antibodies. In applications where aglycosylated full-length antibodies can be used, high-level expression in *E. coli* has been recently reported (47). The major problem with the CHO expression system is the potential instability of some of the proteins expressed. Recent solutions to address this problem have been the use of retroviral vectors to deliver antibody genes, use of expression vectors containing human antirepressor elements (22), or the use of vectors which target integration near the telomeric regions of the chromosomes. If expression of the protein of interest cannot be achieved in *E. coli* or CHO cells, alternative expression systems are available (2, 8, 43, 45). Regardless of the expression system used, current efforts are focused on the integration of bioprocessing approaches with expression systems to improve downstream recovery and purity of the protein of interest.

In summary, we described the successful expression of 5C12 HuMAb in CHO cells, which displayed neutralizing efficiency equal to that produced by the parent 5C12 HuMAb in hybridoma cells. From a production viewpoint, an advantage of using the 5C12-producing CHO cell line is a four- to sevenfold higher production level of the antibody compared to the hybridoma cell line. This process was the next obvious step in our continued effort to develop an effective therapy against HUS, a serious cause of acute kidney failure in children.

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